Assessing the Genetic Basis of Hindlimb and Pelvic Reduction in Squamate Reptiles

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Introduction

Vertebrate fins and limbs have evolved tremendous diversity in form and function since the group's common ancestor 540 million years ago. However, reduction and loss of vertebrate appendages is common across the vertebrate tree[1]. Is there a shared genetic basis to this loss, or does each clade lose appendages in a different way?

Threespine stickleback fish have repeatedly lost their pelvises after colonization of freshwater habitats[1]. This pelvic reduction has been traced to a gene called *Pitx1,* in particular a loss of expression in the pelvis[1]. Crucially for our study, loss of *Pitx1* expression in stickleback (and experimental mice) leaves a phenotypic signature.

That is, when *Pitx1* fails to express in developing hindlimbs, left-side vestigial pelvises tend to be larger than right-side vestiges[1]. Such left-side asymmetry has been observed in several species of wild stickleback and even in manatees, a mammal. This suggests that *Pitx1* could be a common route to pelvic reduction in vertebrates.

Here, we test for this phenotypic signature of *Pitx1*-caused pelvic reduction in seven clades of squamate lizards that have independently evolved hindlimb reduction. Observation of left-biased vestigial asymmetry in squamate hindlimb loss would implicate *Pitx1* in yet another major vertebrate lineage.

Methods

- To determine sample size needed to detect significant deviations from symmetry, we ran *pwr.chisq.test* in the statistical software R. Sample sizes > 60 are required to have power > 0.5. We targeted 80-100 individuals per species
- We identified seven squamate clades that independently showed hindlimb and pelvic reduction, and requested museum specimens *Agamadon anguliceps*, *Bachia intermedia*, *Indotyphlops braminus*, *Ophisaurus attenuatus*, *Ophisaurus ventralis*, *Sphenops sepsoides*, *Pgyopus lepidopodus*, and *Teius teyou*
- Specimens were wrapped in a plastic bag and scanned in a Perkin-Elmer microCT scanner for four minutes with pelvic girdle or femur in view
- Scans were exported to the bone reconstruction program InVesalius v.3.1 to generate 3D images
- Reconstructed images were landmarked with MeshLab 2016 by S.S. to calculate measurements of pelvic girdle and femur
- Landmarks were exported to R for analysis
- For each trait for each species, we used a two-tailed chi-square test (*chi.sq.test* in R) to test if the proportion of specimens with larger left-side vestiges deviated significantly from 50%
- To quantify the magnitude of deviation, we used a two-tailed, paired t-test (*t.test* in R, paired = TRUE) to determine if left-side vestiges were significantly larger than right-side vestiges.

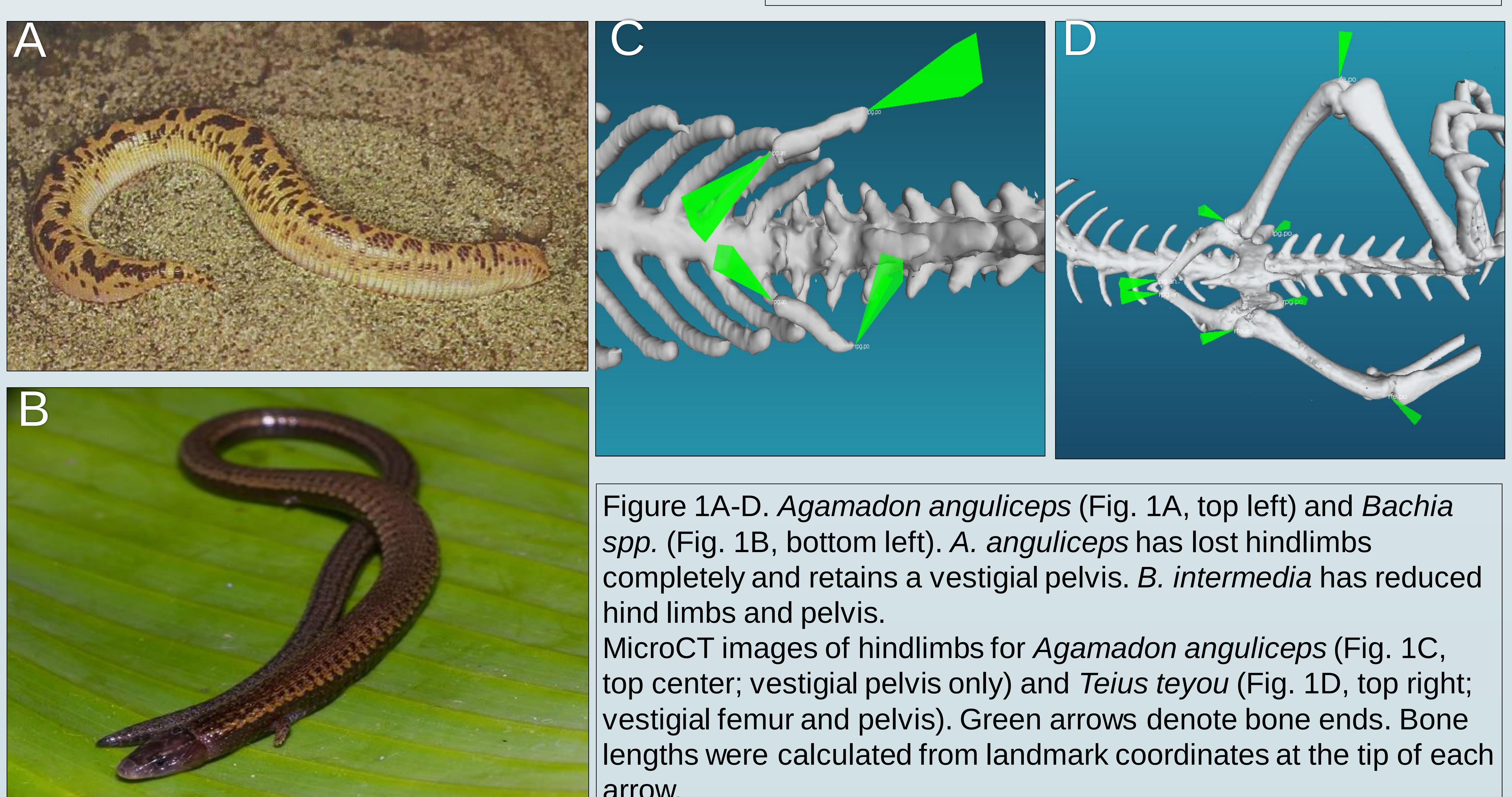
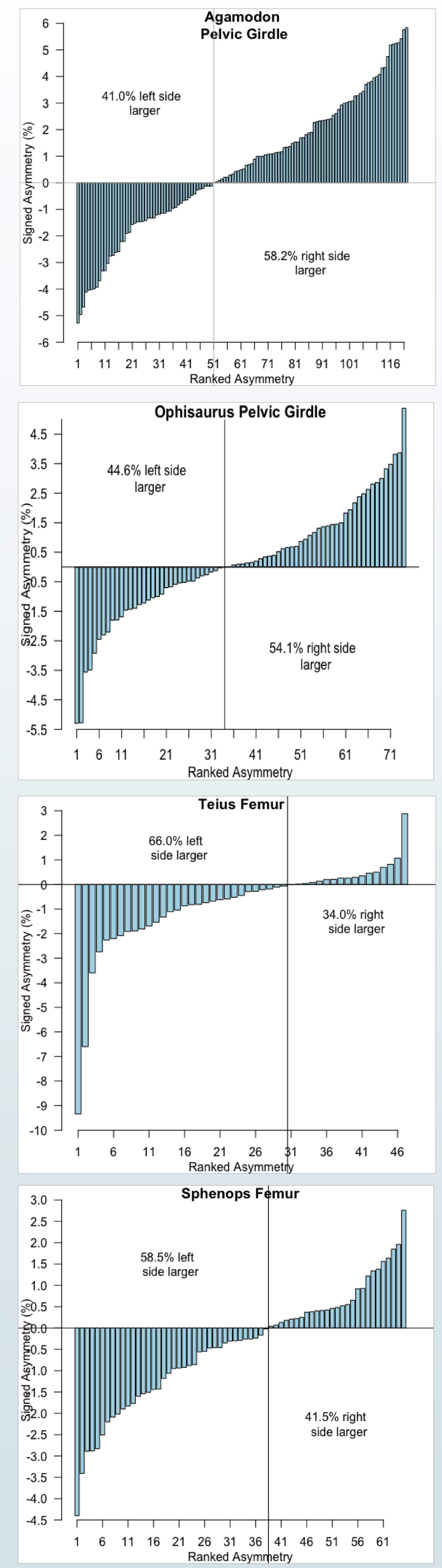


Figure 1A-D. *Agamadon anguliceps* (Fig. 1A, top left) and *Bachia spp.* (Fig. 1B, bottom left). *A. anguliceps* has lost hindlimbs completely and retains a vestigial pelvis. *B. intermedia* has reduced hind limbs and pelvis.
MicroCT images of hindlimbs for *Agamadon anguliceps* (Fig. 1C, top center; vestigial pelvis only) and *Teius teyou* (Fig. 1D, top right; vestigial femur and pelvis). Green arrows denote bone ends. Bone lengths were calculated from landmark coordinates at the tip of each arrow.

Results



Plots of left-vs.-right asymmetry for pelvic and femur vestiges. Individuals are ordered on the x-axis by their asymmetry value. Values below the x-axis are left-side larger. Values above are right-side larger. Vertical line shows the shift from left to right side bias. We find left and right-side bias in our sample, though not always significantly so (Table 1).

Table 1. Results of paired t-tests and chi-square tests, used to detect left-biased asymmetry in hindlimb vestiges. *t*-tests ask whether asymmetry is biased to one side; positive *t*-statistics imply left-biased asymmetry. Chi-square tests probe for deviation from 50:50 left vs. right bias in a sample.

Pelvis	***t*-stat (d.f.)**	**P value**	**X^2 (1 d.f.)**	**P value**
Agamadon anguliceps	-2.5 (122)	0.01	1.65	0.20
Indotyphlops braminus	1.44 (90)	0.15	0.05	0.82
Sphenops sepsoides	1.38 (72)	0.17	0.06	0.80
Teius teyou	-2.14 (47)	0.04	2.1	0.15
Ophisaurus spp.	-0.18 (74)	0.86	0.24	0.62
Femur				
Sphenops sepsoides	2.56 (64)	0.01	0.63	0.43
Teius teyou	2.08 (46)	0.00	1.84	0.17

Discussion

If the left-side larger asymmetry is statistically significant, *Pitx1* may be driving hindlimb and pelvic reduction. The species we examined do not demonstrate a statistically significant deviation toward either right or left-biased asymmetry in the pelvis or femur**.** However, the right pelvic vestiges of *A. anguliceps* and *T. teyou* are on average significantly larger on the left (p = 0.01 and 0.04, respectively), while *I. bramin*us, *O. spp*, and *S. sepsoides*, on average, have pelvises with insignificantly larger right-side remnants (p = 0.15, 0.86, and 0.17, respectively). *S. sepsoides* and *T. teyou* have statistically significant left-larger femurs (p = 0.01 and 0.00, respectively).

Thus, our data do not suggest squamate limb loss is underlain by loss of *Pitx1* expression. This is likely because *Pitx1* is controlled by different regulatory mechanisms in fish and tetrapods: *PelA* is an enhancer unique to fish while *Pen* is found only in tetrapods. Pleiotropy, when one gene influences two or more phenotypic traits, may also constrain *Pitx1* regulation in tetrapods.

We look forward to publishing these data soon as we have a manuscript in progress.

References

1. Shapiro, M., Marks, M., Peichel, C. *et al.* Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* **428,** 717–723 (2004). https://doi.org/10.1038/nature02415
2. Agamadon Anguliceps image: https://reptile-database.reptarium.cz/species?genus=Agamodon&species=anguliceps
3. Bachia intermedia image: https://en.wikipedia.org/wiki/Bachia
4. Thompson, Abbey C et al. "A novel enhancer near the *Pitx1* gene influences development and evolution of pelvic appendages in vertebrates." *eLife* vol. 7 e38555. 30 Nov. 2018, doi:10.7554/eLife.38555

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